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13. SUPPLEMENTARY NOTES

14. ABSTRACT **Subject:** During breast cancer progression and metastasis, tumor cells acquire the ability to survive and grow in stressful microenvironments. The genetic lesions that drive proliferation and prevent cell death during tumor development are well understood, however, less is known about the contributions of pathways that allow cells to cope with environmental stress, such as autophagy. **Purpose:** Although autophagy is known to aid in cell survival in response to a wide range of stress stimuli, it remains unclear whether autophagy enhances or suppresses the development and progression of breast cancer (Mizushima et al., 2008). Understanding the role of autophagy during stress in the context of breast cancer cells will allow us to determine if autophagy could be a valuable drug target for breast cancer treatment. **Scope:** The goals of this research project are to: 1) Determine if breast epithelial cells (MCF10A cells) expressing oncogenes mutated in breast cancer initiate autophagy during extracellular matrix detachment, 2) Determine if autophagy inhibition promotes cell death during extracellular matrix detachment, alters 3D morphogenesis, and contributes to oncogenic transformation, and 3) Determine if autophagy suppression increases cell death and alters transformation in established breast cancer cell lines. To date we have determined that autophagy is induced in breast epithelial cells expressing oncogenes that activate the Ras and PI3K pathways and in MDA-MB-231 cells, a breast cancer cells line. In addition, we have found that autophagy suppression alters 3D morphogenesis and suppresses transformation of oncogene expressing breast epithelial cells.

15. SUBJECT TERMS
autophagy, oncogenes, extracellular matrix detachment, 3D morphogenesis, cell transformation

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ANNUAL STATUS REPORT: BC083204. Detachment-Induced Autophagy and Breast Cancer Cell Survival

INTRODUCTION: **Subject:** During breast cancer progression and metastasis, tumor cells acquire the ability to survive and grow in stressful microenvironments. The genetic lesions that drive proliferation and prevent cell death during tumor development are well understood, however, less is known about the contributions of pathways that allow cells to cope with environmental stress, such as autophagy. **Purpose:** Although autophagy is known to aid in cell survival in response to a wide range of stress stimuli, it remains unclear whether autophagy enhances or suppresses the development and progression of breast cancer (Mizushima et al., 2008). Understanding the role of autophagy during stress in the context of breast cancer cells will allow us to determine if autophagy could be a valuable drug target for breast cancer treatment. **Scope:** The goals of this research project are to: 1) Determine if breast epithelial cells (MCF10A cells) expressing oncogenes mutated in breast cancer initiate autophagy during extracellular matrix detachment, 2) Determine if autophagy inhibition promotes cell death during extracellular matrix detachment, alters 3D morphogenesis, and contributes to oncogenic transformation, and 3) Determine if autophagy suppression increases cell death and alters transformation in established breast cancer cell lines. To date we have determined that autophagy is induced in breast epithelial cells expressing oncogenes that activate the Ras and PI3K pathways and in MDA-MB-231 cells, a breast cancer cells line. In addition, we have found that autophagy suppression alters 3D morphogenesis and suppresses transformation of oncogene expressing breast epithelial cells.

BODY: Below is a summary of results from tasks outlined in the approved Statement of Work that have been completed to date.

Task 1: *Determine if breast epithelial cells expressing activated PI3K and HER2/NEU induce autophagy during ECM detachment.*

Results from Task 1 are described in detail in our year-one status report. In summary we have used both western blot analysis of LC3-II levels (Tasks 1A and 1C) and as well as evaluation of both GFP-LC3 and mCherry-GFP-LC3 puncta (Tasks 1B and 1D-1E) to asses autophagy levels in MCF10A cells expressing PI3KH1047R, NeuT, and RasV12. Taken together these results indicate that autophagy is robustly induced in MCF10A cells expressing PI3KH1047R, NeuT, and RasV12 following extracellular matrix attachment.

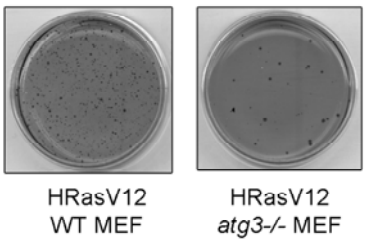
Task 2: *Determine if autophagy inhibition promotes the death of oncogene expressing cells during ECM detachment in both 2D and 3D models, and if autophagy contributes to oncogenic transformation.*

2G: *Assess transformation potential of control, PI3KH1047R, and HER2/NEU expressing wild-type and ATG5/7 null MEFs by quantifying colonies following growth in soft agar.*

In our status report from year one we describe in detail results from experiments comparing soft agar growth of RasV12 wild-type and ATG knockout MEFs. As previously reported, we found that both RasV12 *atg5-/-* and *atg7-/-* MEFs displayed a significant reduction

in soft agar growth as compared to RasV12 wild-type controls. We have now further confirmed this result by examining soft agar growth of RasV12 expressing ATG3 wild-type and deficient MEFs (Figure 1). As we observed with both ATG5 and ATG7 deficient MEFs, we observed a significant decrease in soft agar formation in RasV12 *atg3-/-* MEFs. This in addition to our previous results indicates autophagy is necessary to support robust transformation by RasV12.

Figure 1: Soft agar growth of RasV12 expressing wild-type (WT) and *atg3-/-* MEFs. Representative soft agar plates from RasV12 WT and RasV12 *atg3-/-* MEFs.



Since we had previously observed increases in apoptosis following extracellular matrix detachment in wild-type MCF10A cells in which autophagy was inhibited (Fung et al., 2007) and due to the dramatic decreases in soft agar growth observed with RasV12 ATG knockout MEFs, we decided to use RasV12 expressing *atg5* wild-type and *atg5*-/- MEFs to determine if loss of autophagy would enhance apoptosis during extracellular matrix detachment (one of our goals in Task 2C and D). Although it has been reported that MEFs do not undergo apoptosis following extracellular matrix detachment, we observed an increase in cleaved-caspase 3 levels by western blot following extracellular matrix detachment in RasV12 expressing MEFs. Additionally, the levels of cleaved-caspase 3 increased further in RasV12 *atg5*-/- MEFs, suggesting an increased level of apoptosis in autophagy deficient RasV12 expressing cells following matrix detachment (Figure 2).

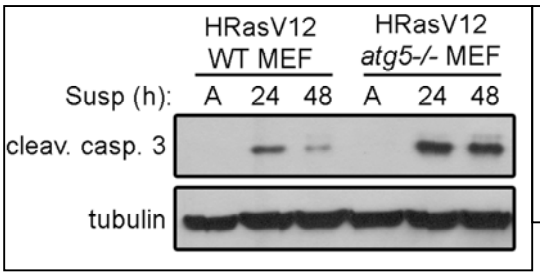


Figure 2: Levels of cleaved-caspase 3 in RasV12 wild-type (WT) and *atg5-/-* MEFs in attached conditions (A) and following 24 and 48 hours suspension (susp).

2B: ***Stably express shot hairpin RNAs against ATG5 and ATG7 in empty vector, PI3KH1047R, and HER2/NEU MCF10A cells and validate knockdown by western blot.***

As described in our first year status report, we were unable to achieve a high level of knockdown using siRNAs against critical autophagy genes (ATGs) which was originally proposed in Task 2A. Thus, we have decided to use short-hairpin RNAs against critical autophagy genes (ATGs) for all of our experiments requiring autophagy knockdown. In our previous report we show data that we were able to stably express shRNAs against ATG7 and ATG12 and achieve a high level of target knockdown in both empty vector and RasV12 expressing MCF10A cells. We have now been able to stably express these same shRNAs in MCF10A cells expressing PI3KH1047R and NeuNT (a constitutively active form of Neu). Similar to what we previously observed using RasV12 cells we were able to achieve the best knockdown using shRNA against ATG7 (Figure 3) whereas knockdown of ATG12 resulted in a much lower level of target knockdown (data not shown). Due to the low level of knockdown achieved with shATG12 we have been focusing on using shATG7 hairpins while we try to improve knockdown efficiency with other shATGs.

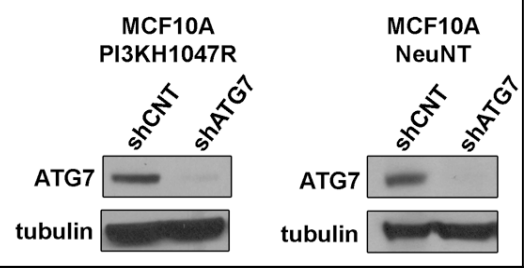


Figure 3: Knockdown of AT G7 in MCF10A PI 3KH1047R and NeuNT cells following shATG7 expression.

2E: *Set-up 3D morphogenesis assays in PI3KH1047R and HER2/NEU MCF10A cell lines.*

We have begun to evaluate the contribution of autophagy during 3D morphogenesis in MCF10A cells expressing PI3KH1047R and NeuNT. Wild-type MCF10A cells will form three-dimensional polarized structures with a hollow lumen when grown on a laminin rich basement membrane (Debnath et al., 2005). The formation of a hollow lumen occurs through the selective apoptosis of cells within the center of the structure. We have previously found that autophagy inhibition results in an increase in luminal apoptosis in wild-type MCF10A structures resulting in a more rapid clearance of the lumen (Fung et al., 2007).

When grown in 3D PI3KH1047R MCF10A cells formed large structures that eventually clumped together following 15 days in culture (data not shown). At earlier time points PI3KH1047R structures appear similar to wild-type structures in that the majority of structures are spherical in shape (Figure 4, top images). Additionally, similar to wild-type structures, PI3KH1047R expressing structures remain polarized. However, unlike wild-type structures the lumens of PI3KH1047R structures remain partially filled. When we inhibited autophagy, by expressing shATG7 in these structures, we did not observe any obvious differences in the overall phenotype of the structures that formed (Figure 4, top images). More specifically, autophagy deficient PI3KH1047R structures appear to be relatively the same size as shCNT structures, become fused together at later timepoints, and maintain a polarized morphology. However, from comparing phase images of MCF10A PI3KH1047R cells expressing shCNT to those expressing shATG7, we noticed larger depressions in the center of shATG7 expressing structures suggesting a potential increase in luminal clearance, which will be discussed further in task 2F.

Expression of NeuNT during 3D morphogenesis resulted in structures that we misshapen and disorganized (Figure 4, bottom images). Unlike RasV12 expressing structures, however, expression of NeuNT is not sufficient to cause the formation of invasive branches. Additionally, unlike wild-type structures, NeuNT structures maintain a completely filled lumen. Expression of shATG7 in NeuNT cells resulted in structures that were slightly smaller than shCNT-expressing structures suggesting a decrease in proliferation of NeuNT shATG7 structures

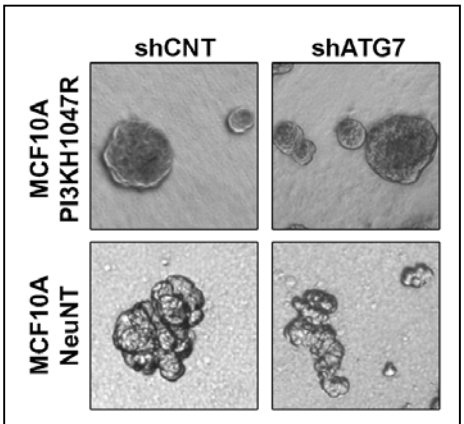


Figure 4: Representative phase images of 3D structures from PI3KH1047R and NeuNT MCF10A cells expressing shCNT or shATG7.

compared to shCNT (Figure 4, bottom images). Although shATG7 expressing NeuNT structures were smaller than shCNT structures, we did not observe a reversal in the misshapen phenotype that occurs following NeuNT expression. This is in contrast to what we observed following autophagy inhibition in MCF10A cells expressing RasV12. As described in detail in our status report from year one, expression of shATG7 in MCF10A RasV12 cells resulted in a dramatic reversal in the invasive phenotype that occurs following RasV12 expression during 3D morphogenesis.

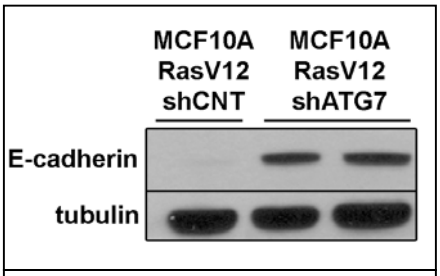
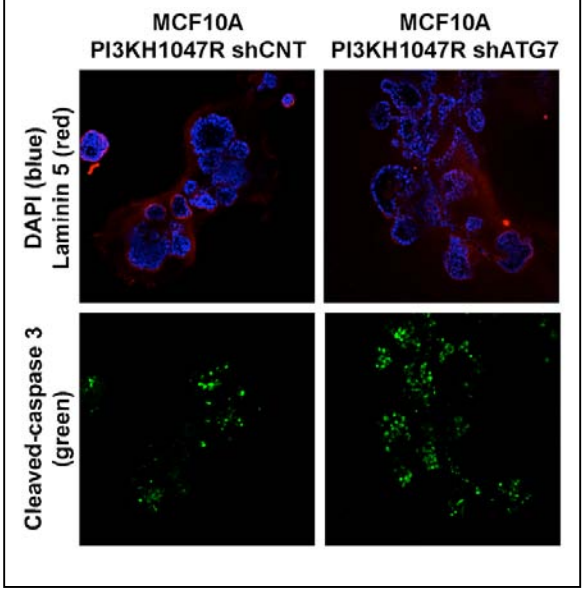


Figure 5: E-cadherin and tubulin levels in RasV12 MCF10A cells expressing shCNT or shATG7

Additional Findings:

We reported last year that inhibition of autophagy in RasV12 MCF10A results in a suppression of invasion during 3D morphogenesis. Furthermore, RasV12 MCF10A cells, which lose polarity and proper cell-cell contacts when grown in 3D, were found to display a restoration in both polarity and cell-cell junctions following autophagy inhibition. We have followed up on this finding and have begun to compare markers known to contribute to cell invasion and migration between MCF10A RasV12 shCNT and shATG7 expressing cells. Interestingly, we found that E-cadherin a critical molecule in mediating cell-cell junctions, which is lost upon RasV12 expression, is partially restored in shATG7 expressing cells in both attached conditions (Figure 5) and in 3D (data not shown). Loss of E-cadherin is often indicative of a highly invasive and migratory phenotype; therefore, the restoration we observe in autophagy-depleted cells may explain the phenotype we observe in 3D. Furthermore, this finding potentially implicates autophagy in promoting later stages of breast cancer as E-cadherin levels are often inversely correlated with tumor severity.

Figure 6: Top: DAPI (blue) and laminin 5 (red) staining of PI3KH1047R shCNT and shATG7 structures. Bottom: Levels of cleaved-caspase 3 (green) in the same structures



2F: *Measure apoptosis (cleaved-cleaved caspase 3) in 3D structures:*

As mentioned above in task 2E while examining phase images of MCF10A PI3KH1047R structures expressing shCNT or shATG7 we noticed darker depressions in the centers of shATG7 structures compared to control. This suggests an increased level of luminal clearance and luminal apoptosis. In fact, when we stained PI3KH1047R shCNT and shATG7 structures with cleaved-caspase 3 we observed an increased level of cleaved-caspase 3 positive cells in shATG7 structures (Figure 6). This result is in accordance with data we have previously published on wild-type MCF10A 3D morphogenesis in which autophagy inhibition leads to enhanced levels of luminal apoptosis.

2H: *Assess transformation potential of MCF10A stable autophagy knockdown lines by quantifying colony formation in soft agar.*

As we have now been able to consistently achieve stable knockdown of essential autophagy genes, we have begun to address the importance of autophagy during soft agar growth in MCF10A cells expressing various oncogenes. We have started by comparing soft agar growth of MCF10A RasV12 cells expressing either shCNT or shATG7. We decided to use RasV12 expressing MCF10A cells, as PI3KH1047R MCF10A cells did not efficiently form colonies in soft agar. RasV12 MCF10A cells formed small colonies in soft agar following two weeks of growth that were visible under low magnification. We therefore took multiple images of each plate at 4x magnification and quantified the number of colonies per plate across three separate experiments. Similar to what was observed in RasV12 MEFs, RasV12 MCF10A cells expressing shATG7 formed significantly fewer colonies, which was clearly evident from the images that were taken. Following quantification of the images, we observed almost a 50% reduction in colony formation in MCF10A RasV12 shATG7 cells compared to shCNT (Figure 7).

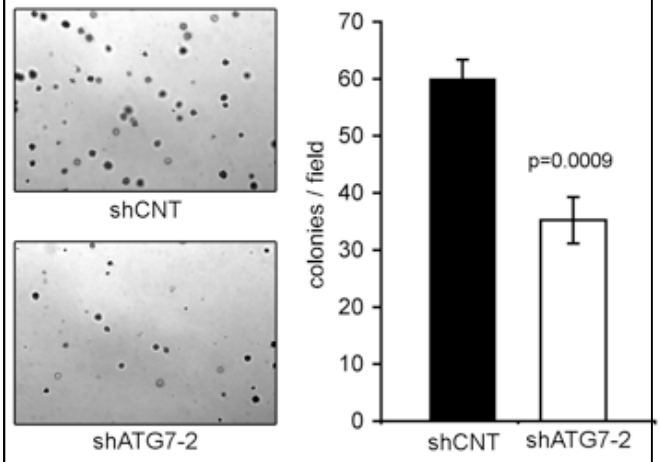


Figure 7: Left: Representative images of MCF10A RasV12 shCNT and shATG7 colonies following growth in soft agar. Right: Quantification of the number of colonies per field following growth of MCF10A RasV12 shCNT and shATG7 in soft agar.

Task 3: *Determine if autophagy suppression increases cell death in established breast cancer cell lines following matrix detachment and during anchorage independent transformation.*

3A: *Examine levels of autophagy by western blot of LC3-I and LC3-II in breast cancer cell lines following suspension.*

We have begun to examine whether autophagy is induced in breast cancer cells lines following extracellular matrix detachment by using MDA-MB-231 cells. These cells were plated on poly-hema coated 6-well plates to prevent attachment and grown for 24h hours. Prior to lysis, half were treated with E64d and pepstatin A, to inhibit degradation of LC3-II and enable us to evaluate degradation of autophagosomes in the lysosome. Lysates from suspended cells were then compared to attached cells both treated and untreated with E64d and pepstatin A. We found MDA-MB-231 cells displayed a robust increase in LC3-II levels following 24h of

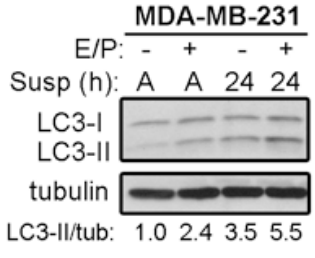


Figure 8: Western blot of LC3 in MDA-MB-231 cells in attached conditions and following 24h suspension (susp). Bottom of blot: levels of LC3-II normalized to tubulin.

suspension and levels of LC3-II were further enhanced following the addition of lysosomal inhibitors (Figure 8). This indicates autophagy is induced in MDA-MB-231 cells following suspension and that the autophagosomes that form are properly degraded in the lysosome.

3B: *Express GFP-LC3 in breast cancer cells lines and measure punctate GFP-LC3 levels following suspension using confocal microscopy.*

Following the observation of robust autophagy during extracellular matrix detachment in MDA-MB-231 cells as described above; we have taken this cell line and stably expressed GFP-LC3. As autophagy is induced GFP-LC3 relocalizes from the cytoplasm to autophagosomes resulting in the visualization of GFP positive "puncta" within the cell. This assay is commonly used in combination with western blots for LC3-II to determine autophagy levels. MDA-MB-231 cells expressing GFP-LC3 were plated attached or on poly-hema coated plates, to prevent cell attachment, for 24h. Cells were then fixed and mounted on coverslips to image GFP positive puncta. As we expected from the results described above in Task 3A, we observed a large increase in GFP positive puncta following matrix detachment in MDA-MB-231 cells. Due to the aggregation that occurs following suspension, we are unable to quantify the precise levels of GFP puncta per cell, however, we feel it is clear from the images there is a robust induction of autophagy following extracellular matrix detachment in MDA-MB-231 cells.

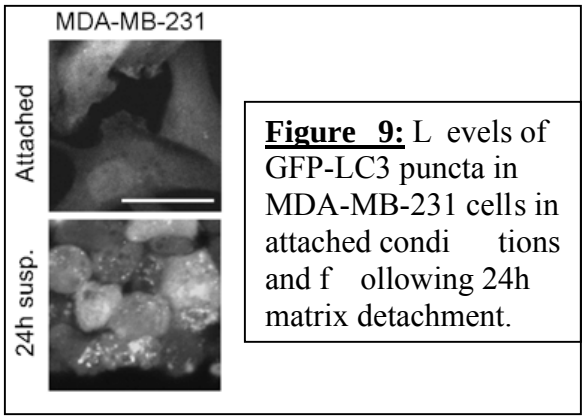


Figure 9: Levels of GFP-LC3 puncta in MDA-MB-231 cells in attached conditions and following 24h matrix detachment.

KEY RESEARCH ACCOMPLISHMENTS:

- Generation of PI3KH1047R and NeuNT MCF10A cells expressing short-hairpins against critical autophagy genes.
- Characterization of 3D morphogenesis phenotypes resulting from autophagy inhibition in PI3KH1047R and NeuNT expressing MCF10A cells.
- Evaluation of cleaved-caspase 3 levels (apoptosis levels) in PI3KH1047R MCF10A cells expressing shATG7 during 3D morphogenesis.
- Western blot analysis of autophagy induction in MDA-MB-231 cells following 24h extracellular matrix detachment.
- Generation of MDA-MB-231 cells expressing GFP-LC3. Confirmation of autophagy induction following 24h suspension in MDA-MB-231 cells expressing GFP-LC3.

REPORTABLE OUTCOMES:

Publications:

Lock, R., Roy, S., Kenific, C., Su, J., Salas, E., Ronen, S., and Debnath, J. **Autophagy Facilitates Glycolysis During Ras Mediated Oncogenic Transformation.** In revision.

Lock, R. and Debnath, J. **Extracellular matrix regulation of autophagy.** Curr Opin Cell Biol. 2008 Oct;20(5):583-8.

Abstracts:

1. Autophagy inhibition alters glucose metabolism and promotes epithelial differentiation during Ras-mediated oncogenic transformation. Rebecca Lock, Candia Kenific, Srirupa Roy, Eduardo Salas, and Jayanta Debnath. Keystone Symposia: Cell Death Pathways Apoptosis, Autophagy and Necrosis / Metabolism and Cancer Progression. March 12-17, 2010.

- Short talk presenter
- Poster presenter

2. Autophagy inhibition alters glucose metabolism and promotes epithelial differentiation during Ras-mediated oncogenic transformation. Rebecca Lock, Candia Kenific, Srirupa Roy, Eduardo Salas, and Jayanta Debnath. Gordon Conference on Autophagy in Stress, Development and Disease. April 25-30, 2010.

- Poster presenter

CONCLUSION:

Since the funding of this award, we have determined that autophagy is induced in breast epithelial cells (MCF10A) expressing oncogenes that activate the PI3K and Ras pathways both by western blot analysis of LC3-II levels and by visualization of GFP-LC3 and mCherry-GFP-LC3 puncta formation. Additionally, we have observed robust autophagy induction in MDA-MB-231 cells, an established breast cancer cell line. This result is important in understanding the role of autophagy in breast cancer development as it has been assumed that autophagy is suppressed during breast cancer development particularly through activating mutations in the PI3K and Ras pathways that have previously been thought to suppress autophagy induction.

Next, we have validated short-hairpin RNAs against autophagy genes that are able to not only achieve knockdown of the intended targets, but also significantly inhibit autophagy induction. These shRNAs are critical to evaluating the contribution of autophagy during 3D morphogenesis and transformation in oncogene expressing MCF10A cells as well as the breast cancer cell lines we intend to use in Task 3. Using these cells, we have found that RasV12 MCF10A cells expressing shATG7 display a significant reduction in soft agar growth compared to controls indicating an important role for autophagy in oncogenic transformation of breast epithelial cells.

Finally, we have begun to examine the role of autophagy during 3D morphogenesis of oncogene expressing breast epithelial cells using RasV12 MCF10A, PI3KH1047R MCF10A, and NeuNT MCF10A cells expressing shATG7. Using RasV12 MCF10A cells we have found that although autophagy inhibition does not cause an increase in the levels of apoptosis during 3D morphogenesis, as originally hypothesized, we found that autophagy inhibition was sufficient to decrease both the growth and invasiveness of RasV12 MCF10A structures, which corresponds with a partial restoration in polarity and cell-cell contacts. Recently we have found that RasV12 MCF10A cells expressing shATG7 display a partial restoration in the cell-cell junction protein E-cadherin that may explain the decreased invasion observed during 3D morphogenesis. In

contrast, we have found that PI3KH1047R MCF10A cells expressing shATG7 cells form 3D structures that are indistinguishable from shCNT in both size and shape. However, shATG7 structures display an enhanced level of apoptosis during 3D morphogenesis. These results suggest the function of autophagy is highly context dependant and might be altered depending on the oncogenic insult present. In the case of RasV12 3D morphogenesis our preliminary results suggest a potential role for autophagy in contributing to invasive and metastatic potential during tumorigenesis. In contrast, in the case of an activating PI3K mutation, autophagy appears to be critical for the survival of luminal cells. Overall, these results will contribute to an increased understanding of how autophagy might be modulated during breast cancer development and how it might enhance breast cancer progression.

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Fung, C., Lock, R., Gao, S., Salas, E., and Debnath, J. (2007). Induction of autophagy during extracellular matrix detachment promotes cell survival. Mol. Biol. Cell. *19*, 797-806.

Debnath, J., and Brugge, J.S. (2005). Modeling glandular epithelial cancers in three-dimensional cultures. Nat. Rev. Cancer *5*, 675-688.

APPENDICES:

None.

SUPPORTING DATA:

Embedded in text.